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On-line screening methods for antioxidants scavenging superoxide anion radical and hydrogen peroxide by liquid chromatography with indirect chemiluminescence detection

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Abstract

The identification of radical species is possible by the electron spin resonance technique. However, the antioxidants in complex matrices such as biological and food samples are difficult to determine. Hence, we developed novel screening systems for antioxidants, which are mainly eliminating superoxide anion radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2), by HPLC with luminol-based chemiluminescence (CL) detection. When the sample contains antioxidants, inhibited peaks corresponding to each antioxidant are observed on the chromatogram. The antioxidant activities of catechins and flavones were determined with flow injection analysis by the proposed indirect CL. The scavenging activity for H_2O_2 and $O_2^{-\bullet}$ were different from each catechin and flavone. Furthermore, the potential was dependent upon the number and the position of OH functional group in the structure. Some applications such as the screening of antioxidants in tea products were also investigated. In spite of many peaks appeared on the chromatogram at UV detection, only the peaks corresponding to the compounds having elimination effect to $O_2^{-\bullet}$ and/or H_2O_2 were detected as inhibited peaks. Consequently, the proposed HPLC-CL seems to provide new screening systems for antioxidants possessing inhibition activity of $O_2^{-\bullet}$ and H_2O_2.

Keywords: Antioxidants; Catechins; Flavones; Superoxide anion radical; Hydrogen peroxide; Luminol-based chemiluminescence; Reversed-phase liquid chromatography

1. Introduction

Superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxy radical ($^{\bullet}OH$), which are active oxygen species, are thought to be linked to onset of various pathological conditions such as rheumatoid and cancer [1–5]. These active oxygen species play important role to oxidative decomposition of invaders such as bacteria. The harmful radicals overproduced are eliminated in biological systems by some enzymes possessing antioxidant activity such as superoxide dismutase, catalase, and peroxidase. Non-enzymatic substances in-

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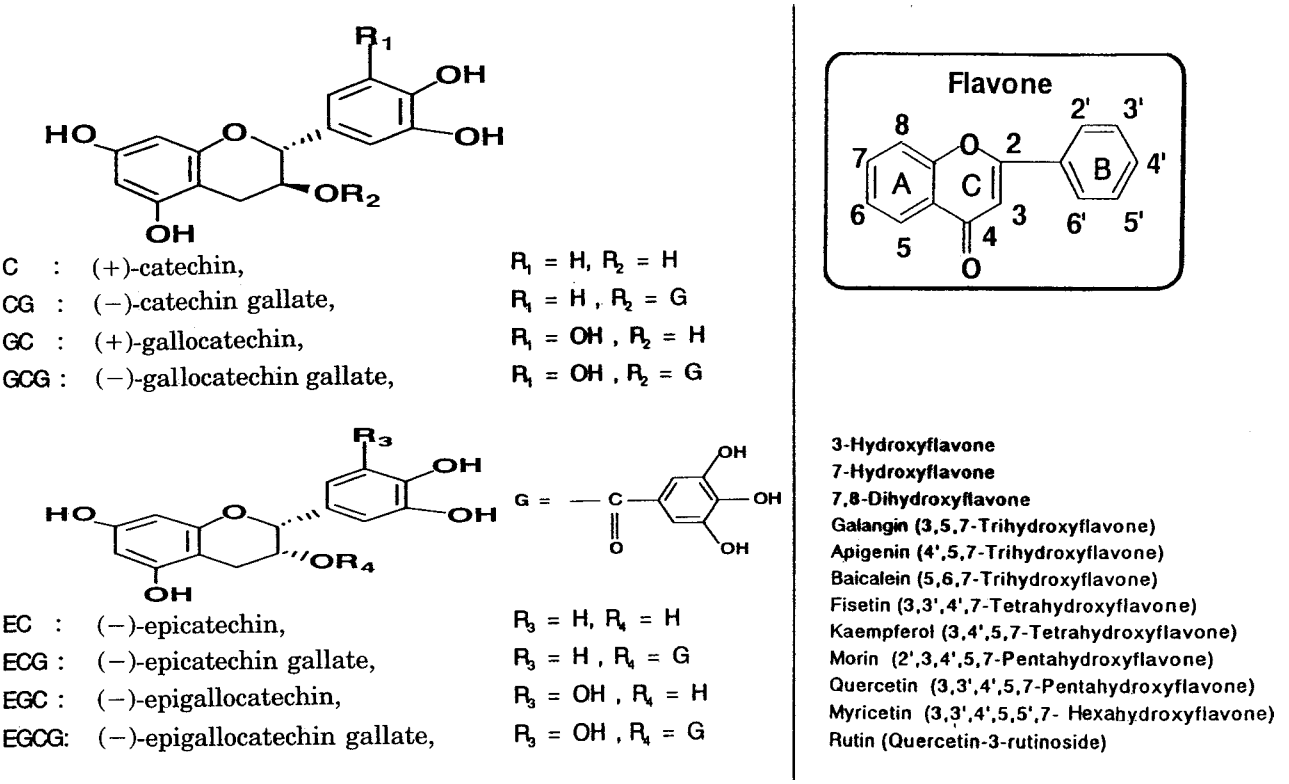

Fig. 1. Structures of catechins and flavones tested.

cluded in diets, e.g., vitamins and flavonoids, also scavenge the free radicals [6–11]. However, it is not obvious what kind of radicals are eliminated with the substance having antioxidant activity. Furthermore, the quantitative assay of antioxidant activity is difficult due to the short life times of these radicals.

Antioxidants have been efficiently resolved by electron spin resonance using spin trapping agents such as DEMPO and TEMPO [12–14]. Although the radical species are possible to identify by the method, the antioxidants in complex matrices such as biological and food samples are difficult to determine in detail. Thus, the antioxidant potential of individual substances is actively investigated and several methods including the use of chemiluminescence (CL) reaction have been developed as sensitive assay [15–19]. The methods only detect antioxidation potential of the substrate. However, the activity toward each radical species could not be determined by the reported procedures.

Table 1
Components of CL reagent solutions and their flow rates for FIA

Solution	Flow rate (ml min^{-1})	Component
FIA for antioxidant scavenging H_2O_2		
I	1.0	4.16 μM luminol
II	1.0	4 mM H_2O_2
Eluent	0.5	Methanol/water (3:7)
FIA for antioxidant scavenging $O_2^{-\bullet}$		
I	1.0	4.l6 μM luminol 3.12 mM HX
II	1.0	3.58 U l^{-1} XOD 15 000 U l^{-1} catalase
Eluent	0.5	Methanol/water (3:7)

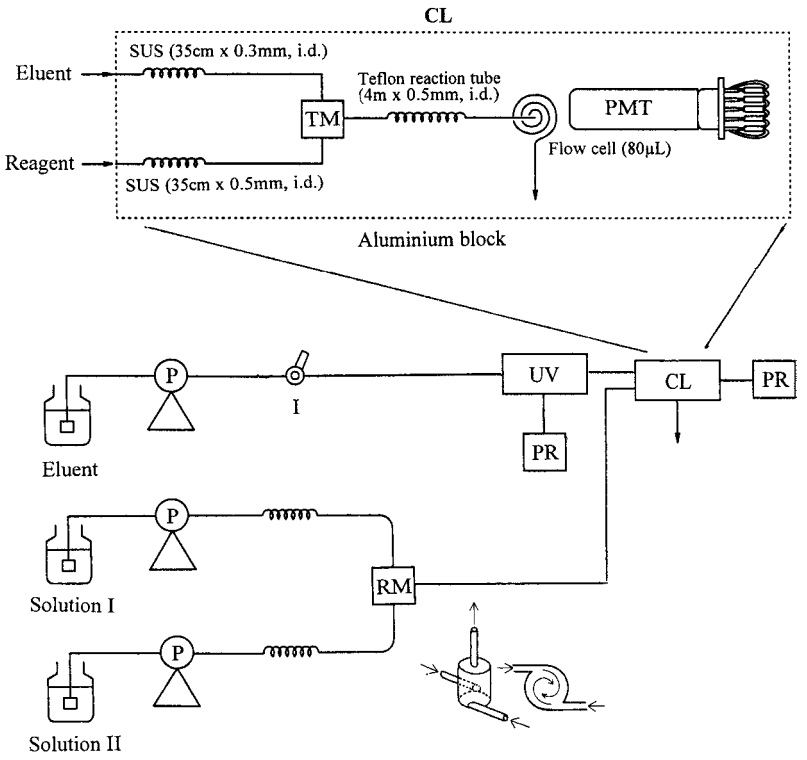

Fig. 2. Schematic flow diagram of HPLC-CL detection. P, pump; I, injector; UV, UV detector; TM, T-type mixing device; RM, rotating mixing device; PMT, photomultiplier; CL, chemiluminescence detector; PR, printer.

In this paper, we present novel assay systems of scavenge activity of antioxidant to the radicals, i.e., $O_2^{-\bullet}$ and H_2O_2, by HPLC with indirect

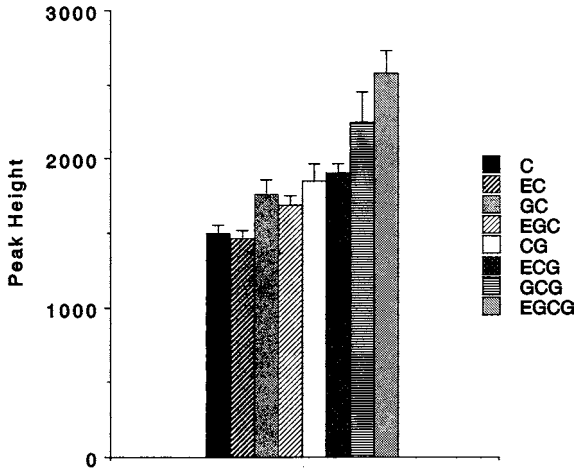

Fig. 3. Antioxidant activity of catechins (25 pmol each, $n = 5$) against hydrogen peroxide.

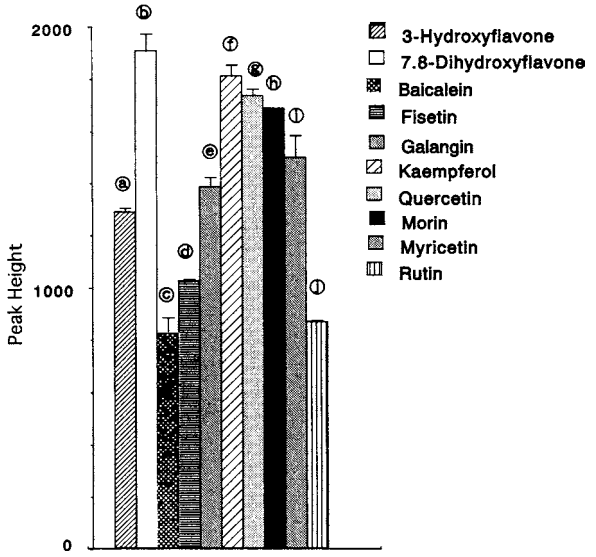

Fig. 4. Antioxidant activity of flavones (25 pmol each, $n = 5$) against hydrogen peroxide.

luminol-based CL. The proposed methods are also applied to the determination of antioxidants in tea samples.

2. Experimental

2.1. Materials and reagents

Luminol, hydrogen peroxide (30% H_2O_2 in water) and hypoxanthine (HX) were obtained from Wako Pure Chemicals (Tokyo, Japan). Enzymes, xanthine oxidase (XOD) (from buttermilk, EC 1.2.3.2, 5 U ml^{-1}) and catalase (from bovine liver, EC 1.11.1.6, 40 000 U mg^{-1} protein), were also purchased from Wako. Flavones, i.e., baicalein, 7,8-dihydroxyflavone, fisetin, galangin, 3-hydroxyflavone, kaempferol, morin, myricetin, quercetin, and rutin, were used as received from Sigma (St. Louis, MO) (Fig. 1). (+)-Catechin (C); (−)-catechin gallate (CG); (+)-gallocatechin (GC); (−)-gallocatechin gallate (GCG); (−)-epicatechin (EC); (−)-epicatechin gallate (ECG); (−)-epigallocatechin (EGC) and; (−)-epigallocatechingallate (EGCG), were generously gifted from Tokyo Food Techno Co. (Tokyo, Japan). Methanol (MeOH) was of HPLC grade (Wako). Deionized and distilled water was used throughout. All other chemicals were of analytical-reagent grade and were used without further purification.

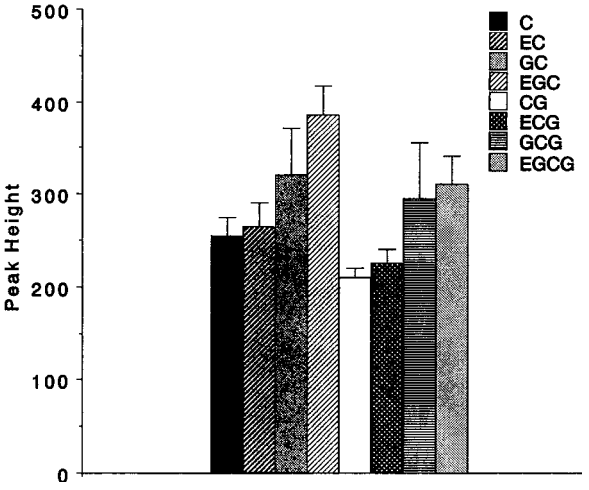

Fig. 5. Antioxidant activity of catechins (25 pmol each, $n = 5$) against super oxide anion radical.

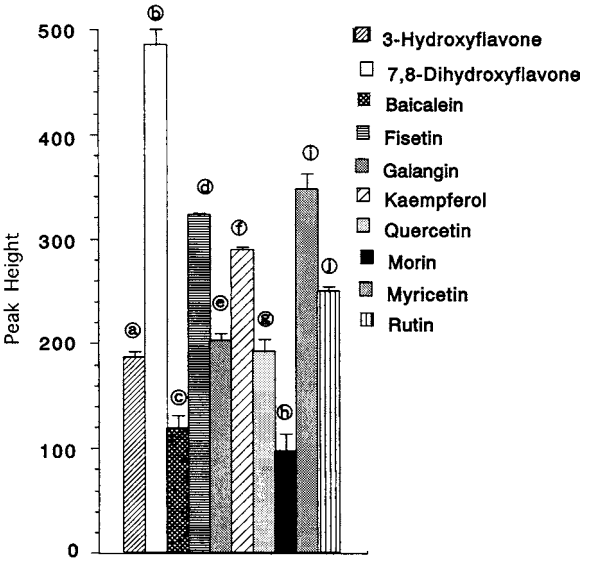

Fig. 6. Antioxidant activity of flavones (25 pmol each, $n = 5$) against super oxide anion radical.

2.2. Reagent solutions

0.2 M phosphate buffer (pH 8.0) was prepared by mixing of appropriate volumes of 0.2 M $NaH_2PO_4 \cdot 2H_2O$ and 0.2 M $Na_2HPO_4 \cdot 10H_2O$. Twenty-five milliliter of 7 mM luminol stock solution was prepared from 1.65 g of sodium hydroxide (NaOH), 1.55 g of boric acid, and 31 mg of luminol. The stock solution was stored in a refrigerator at least 3 days before dilution.

2.2.1. Reagent solutions for the determination of H_2O_2 scavenging activity

Reagent solution I (4.16 μM luminol) was prepared by mixing 7 mM luminol and 0.2 M phosphate buffer (pH 8.0)/MeOH/water (187:40:172). Reagent solution II(4 mM H_2O_2) was prepared from 30% H_2O_2 with dilution of water.

2.2.2. Reagent solutions for the determination of $O_2^{-\bullet}$ scavenging activity

Reagent solution I (mixture of 4.16 μM luminol and 3.12 mM HX) was prepared by mixing 7 mM

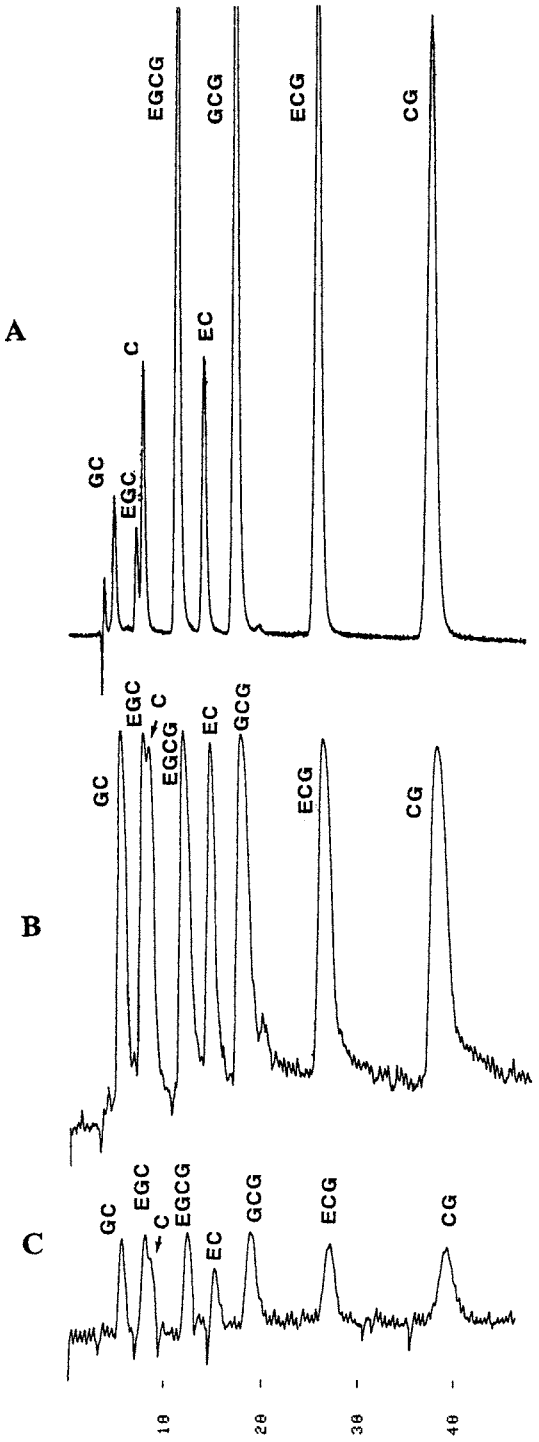

Fig. 7. Chromatograms of authentic catechin mixture. (A) UV detection, (B) CL detection for hydrogen peroxide elimination, (C) CL detection for super oxide anion radical elimination.

luminol, HX and 0.2 M phosphate buffer (pH 8.0)/MeOH/water (469:100:431). The solution II was enzyme mixture of XOD (final concentration, 3.58 $U\,l^{-1}$) and catalase (final concentration, 15 000 $U\,l^{-1}$).

All the solutions including eluent were filtered through by 0.45 μm membrane filter (TYPE HV, Millipore).

2.3. On-line HPLC-CL detection of antioxidants

A Shimadzu LC-10A chromatographs and a SPD-6A UV detector (Kyoto, Japan) was used for separation and detection of antioxidants. The reagent solutions were continuously flowed by two Shimadzu LC-6A pumps. A Shimadzu CLD-10A CL monitor equipped with a 80-μl flow cell was used for the detection of the antioxidants. The signals obtained from UV detection at 265 nm and CL detector were recorded on a Shimadzu C-R6A and a Hitachi 833A Data Processor, respectively. The eluent and the reagent solutions were degassed by sonication, prior to use. The reagent compositions for flow injection analysis (FIA) are shown in Table 1. The eluent for FIA of antioxidants was methanol/water (3:7). The flow rates of the eluent, the reagent solutions, I and II for FIA analysis were 0.5, 1.0, and 1.0 $ml\,min^{-1}$, respectively.

The separation of antioxidant mixture (C, CG, GC, GCG, EC, ECG, EGC, and EGCG) was carried out by J'sphere ODS-H80 (150×4.6 mm I.D., 5 μm) with methanol/water (28:72) containing 1% H_3PO_4. After UV detection at 265 nm, the mobile phase was neutralized with 41.67 mM NaOH solution (Reagent solution III) via a mixing device. The negative peaks obtained from the CL detector were recorded as the positive peaks with phase exchange.

2.4. Pre-treatment of tea samples

Green tea leaves (*Thea sinensis* L.) harvested in Shizuoka prefecture were obtained from a commercial source. The dried tea leaves (1.0 g) were extracted with 100 ml of hot water at 80 °C for 10 min. After cooling at around room temperature (≈ 20 °C), the leaves were filtered out by filter

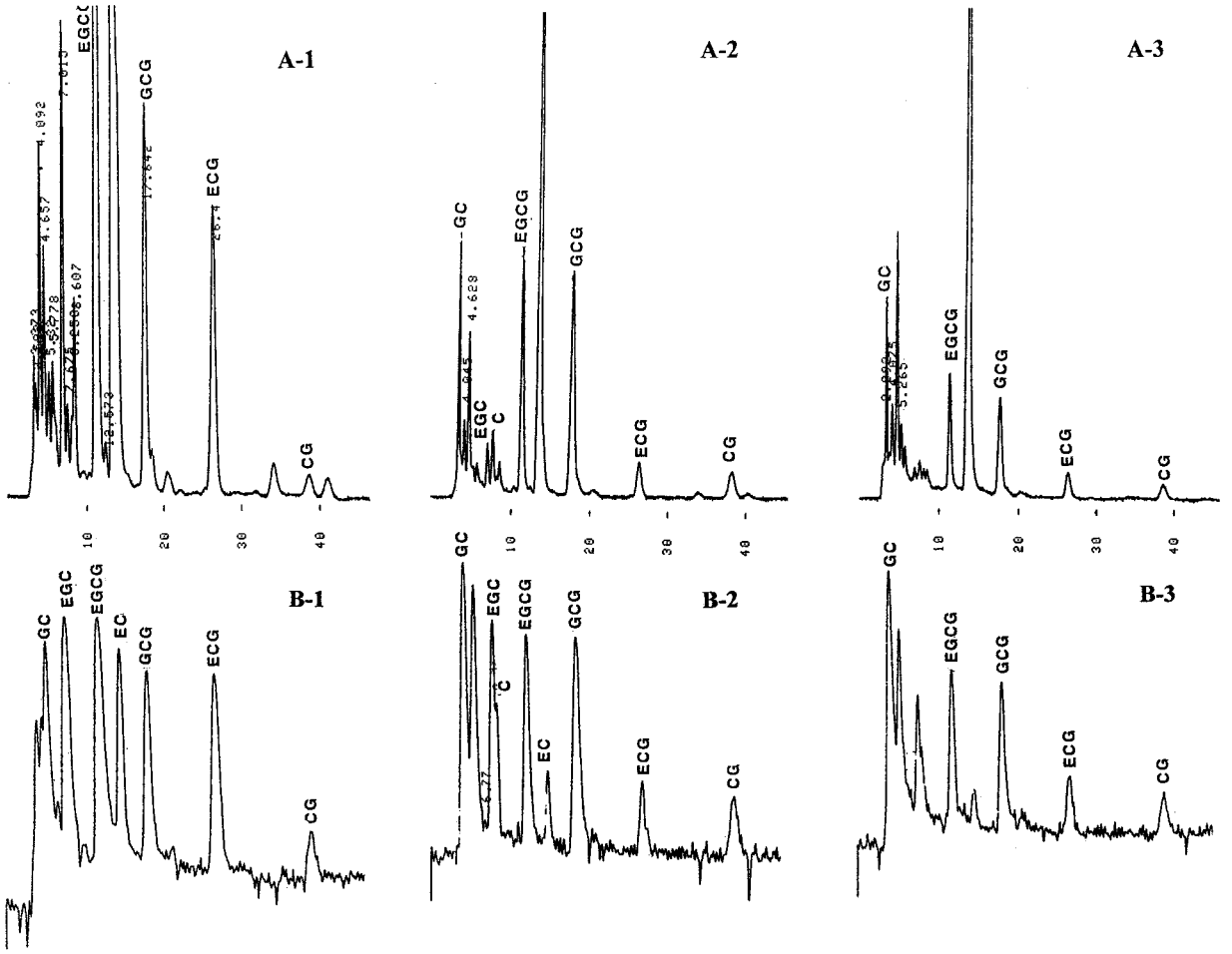

Fig. 8. Chromatograms with UV at 265 nm detection and CL detection of catechins based upon hydrogen peroxide elimination. (A) UV detection, (B) CL detection. *Samples:* A-1 and B-1, green tea extract; A-2 and B-2, green tea product; A-3 and B-3, oolong tea product.

paper, and then the filtrate was centrifuged at 3000 rpm for 15 min. Then, the cloudy solution was passed through a 0.45 μm membrane filter (TYPE HX) under reduced pressure. The solution was diluted to appropriate concentration (10 times) with water. The tea solution diluted was separated by HPLC and determined with UV and CL detectors. The sample pre-treatment was carried out just before analysis.

Green tea and oolong tea products on market were also filtered through the membrane, diluted with water, and analyzed by HPLC-CL, as same as the procedures for green tea leaves.

3. Results and discussion

Novel screening methods for antioxidants by HPLC based upon on-line CL detection were developed. H_2O_2 is detected with chemiluminometry using chemilumigenic reagents such as luminol in alkaline medium. The luminescence is constantly generated from the chemical reaction of luminol and H_2O_2. The CL intensity is dependent upon the concentrations of luminol and H_2O_2. Although higher CL intensity is obtained with the increasing amount of reagents, appropriate concentrations are required for sensitive detection of

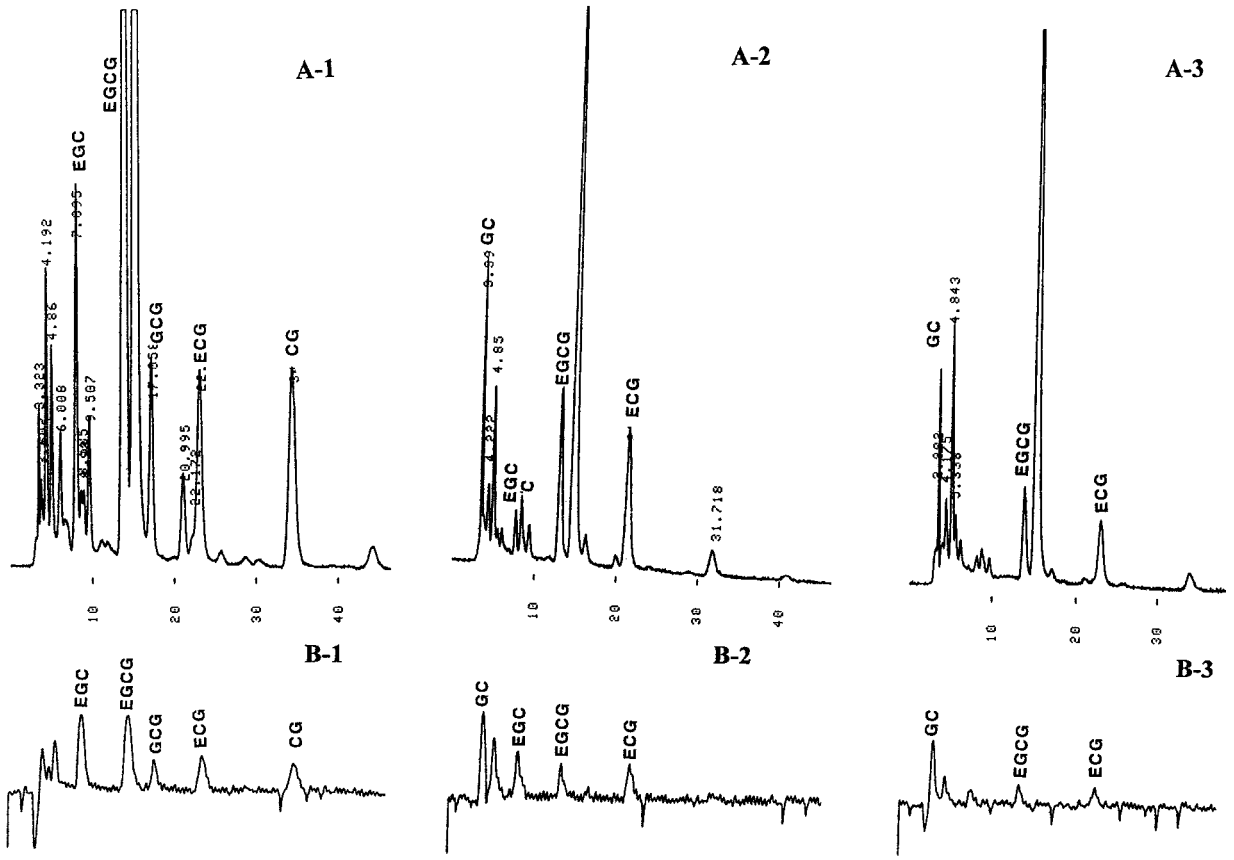

Fig. 9. Chromatograms with UV at 265 nm detection and CL detection of catechins based upon super oxide anion radical elimination. (A) UV detection, (B) CL detection. *Samples:* A-1 and B-1, green tea extract; A-2 and B-2, green tea product; A-3 and B-3, oolong tea product.

antioxidants because high concentrations light saturate the photomultiplier of the CL detector. When the same concentrations of CL reagents (luminol and H_2O_2) are supplied by the on-line HPLC-CL detection system, constant luminescence is produced and recorded as background emission. The background emission decreases when an antioxidant in sample eliminates the H_2O_2, and the baseline is observed as a gap. Furthermore, the degree of activity seems to be proportional to the areas of the inhibited peak.

The CL reaction for the determination of the scavenging activity of H_2O_2 was optimized with FIA without an analytical column. Fig. 2 shows the schematic flow diagram of the proposed on-line HPLC-CL detection. From the results of the optimization study, 4.16 μM luminol and 4.0 mM H_2O_2 were selected for the screening system of H_2O_2 elimination. The flow rates of eluent and the reagent solutions (I and II) were listed in Table 1.

Luminol also emits light from the chemical reaction with $O_2^{-\bullet}$ generated from enzyme reaction of XOD and HX. Antioxidative activity of substances is possible to determine from the degradation of $O_2^{-\bullet}$ concentration. However, the determination of $O_2^{-\bullet}$ contents is very difficult in manual method because of the short life time of $O_2^{-\bullet}$. Thus, the on-line HPLC-CL method for the determination of $O_2^{-\bullet}$ elimination potential was developed (Fig. 2).

The height of baseline by CL detection decreased with the addition of catalase to the reagent solution II. Judging from the results with and without catalase, $O_2^{-\bullet}$ may be rapidly converted

to H_2O_2. Therefore, the enzyme reaction has to take place just before the CL detector to obtain only emission from $O_2^{-\bullet}$ (Fig. 2). From the results of the optimization, the final concentrations recommended for luminol, HX and XOD were 4.16 μM, 3.12 mM and 3.58 U l^{-1}, respectively. Excess amounts of catalase (15 000 U l^{-1}) were added to XOD solution to remove the effect of H_2O_2. The components of the reagent solutions, I and II, and their flow rates are shown in Table 1. When the sample contains antioxidants eliminating $O_2^{-\bullet}$ and/or H_2O_2, down peaks corresponding to each antioxidant are observed on the chromatogram.

The antioxidant activities of catechins and flavones reported as a series of antioxidants were determined with the proposed HPLC-CL systems. The inhibited peaks were observed with all the compounds tested by FIA. As shown in Fig. 3, the order of scavenging potential of catechins toward H_2O_2 was EGCG > GCG > ECG > CG > GC > EGC > C > EC. According to the results, gallate and pyrogallol groups in the catechin structure seem to be important to the elimination of H_2O_2. However, the effect of stereo structure was negligible for the elimination activity, judging from the comparison of the activity between C and EC or GCG and EGCG. Fig. 4 shows the elimination potential of flavones to H_2O_2. In the case of flavones, 7,8-dihydroxyflavone scavenged H_2O_2 most strong among the other flavones. Kaempferol, quercetin, morin and myricetin which have phenolic hydroxyl groups at 3, 5, 7 positions in the structures also scavenged H_2O_2 efficiently.

With respect to the scavenging activity of $O_2^{-\bullet}$, GC and EGC possessing pyrogallol structure were strong, as comparing with the gallate bearing catechins such as CG and ECG (Fig. 5). The activities of GCG and EGCG having both gallate and pyrogallol groups in the structure were stronger than those of C and EC, but lower than those of GC and EGC. Hence, the scavenging effect seems to be dependent upon the substituents binding on 2 and 3 position. On the other hand, 7,8-dihydroxyflavone, myricetin, fisetin and kaempferol provide strong effect, and the scavenging potential was in this order (Fig. 6). Since these flavones possess catechol and pyrogallol groups in the structure, the scavenging ability seems to be due to the number of phenolic hydroxyl groups and their position in the structure.

As the application to real samples, the determination of antioxidants in green tea extracts and tea products were carried out with the proposed online HPLC-CL. Before the analysis of real samples, the simultaneous separation of eight catechins was tried by reversed-phase liquid chromatography using ODS column. Since catechins were relatively unstable in neutral and alkaline solutions, an acidic mobile phase, methanol/water (28:72) containing 1% H_3PO_4, was selected for the separation. After the separation, the eluent was neutralized with sodium hydroxide solution (Reagent solution III) after UV detection because the CL reaction proceeds in neutral to slight alkaline medium. Fig. 7 shows the typical chromatograms of authentic catechin mixture. Although the separation between C and EGC was poor under the elution condition, the other catechins were satisfactorily separated.

The proposed separation and detection methods by HPLC-CL were applied to the determination of antioxidants scavenging $O_2^{-\bullet}$ or H_2O_2 in green tea extracts and tea products. As shown in Fig. 8A and Fig. 9A, various compounds appeared on the chromatogram utilizing UV detection at 265 nm. However, only antioxidants eliminating $O_2^{-\bullet}$ and H_2O_2 were identified on the chromatograms with the indirect CL detection (Fig. 8B and Fig. 9B). The green tea and oolong tea solutions in PET bottle were also analyzed with the HPLC-CL methods. From the chromatographic comparison shown in Figs. 8 and 9, the green tea on market contained C together with the other catechins, whereas only three catechins, i.e., GC, GCG, and EGCG, were detected in oolong tea solution. The results suggest that the proposed methods are selective for the antioxidants.

In conclusion, the proposed HPLC-CL methods seem to provide new assay systems for antioxidants possessing scavenging effect of $O_2^{-\bullet}$ and H_2O_2. The proposed screening method using online HPLC-CL seems to be useful for the detection of antioxidants because of its high selectivity. The method is advantageous for the determination of individual antioxidants in complex matrices. Since tea extract contains various antioxidants which

eliminate active oxygen species, the development of assay system of antioxidants scavenging $^{\bullet}OH$ is also another important topic for understanding elimination mechanism of various active oxygen. Consequently, on-line screening system for the compounds scavenging hydroxy radical is currently investigated in our laboratory.

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